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ECBC-TR-375

**TESTING OF THE BIO-SEEQ®
(SMITHS DETECTION HANDHELD PCR INSTRUMENT):
SENSITIVITY, SPECIFICITY, AND EFFECT OF INTERFERENTS
ON *BACILLUS* ASSAY PERFORMANCE**

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14. ABSTRACT Smiths Detection-Edgewood (SDE), Inc., recently developed a handheld PCR instrument, the Bio-Seeq®, an updated, redesigned version of the small, portable PCR instrument previously known as the Handheld Advanced Nucleic Acid Analyzer (HANAA). The SDE developed the Bio-Seeq® to provide a portable platform for first responders to use for detecting biological threats in civilian areas. To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. In previous work, SDE obtained probe and primer sequences constituting an assay for a gene present in <i>Bacillus anthracis</i> , the causative agent of anthrax. The SDE incorporated this probe and primer set into dried reagent beads, which also contain reagents required for an internal control. These, in turn, are part of a self-contained sampling device that contains buffer and the PCR reagent beads. This report details an analysis of the assay for <i>B. anthracis</i> , including the sensitivity and specificity of the assay, and the effect of some common nontarget ("interferent") materials on the performance of the assay.					
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PREFACE

The work described in this report was authorized under the Cooperative Research and Development Agreement, Project No. 0309C, between the U.S. Army Edgewood Chemical Biological Center and Smiths Detection-Edgewood. This work was started in September 2003 and was completed in January 2004.

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**TESTING OF THE BIO-SEEQ®
(SMITHS DETECTION HANDHELD PCR INSTRUMENT):
SENSITIVITY, SPECIFICITY, AND EFFECT OF INTERFERENTS
ON *BACILLUS* ASSAY PERFORMANCE**

1. INTRODUCTION AND BACKGROUND

In May 2003, the U.S. Army Edgewood Chemical Biological Center (ECBC) and Smiths Detection-Edgewood, Inc. (SDE) entered into a Cooperative Research and Development Agreement (CRADA Project 0309C) for the purpose of developing assays for SDE's handheld PCR instrument, the Bio-Seeq®. SDE developed the Bio-Seeq® to provide a portable platform for use by first responders to detect biological threats in civilian areas. The Bio-Seeq® is an updated, redesigned version of a small, portable PCR instrument previously known as HANAA, Handheld Advanced Nucleic Acid Analyzer.^{1,2} At least one reference to its use in detecting a BW agent exists in the peer-reviewed literature.³ Information on the Bio-Seeq® is available from the manufacturer at <http://63.89.158.169/products/Default.asp?ProductID=6>.

To be ready for introduction into the marketplace, the instrument must be accompanied by a menu of reagents that will enable the user to detect the presence of pathogens in environmental samples. Assays that are designed for any instrument that can accommodate real-time fluorogenic PCR can most likely be adapted for use with the Bio-Seeq®. Molecular biologists at ECBC have experience in the development of real-time fluorogenic PCR assays (TaqMan) for pathogen detection.

In previous work, SDE obtained probe and primer sequences constituting an assay for a gene present in *Bacillus anthracis*, the causative agent of anthrax. SDE incorporated this probe and primer set into dried reagent beads, which also contain reagents required for an internal control. These in turn are part of a self-contained sampling device that contains buffer and the PCR reagent beads.

2. OBJECTIVE

The objective of the work reported here was to answer the following questions:

- a. Is the assay (the probe and primer set) specific for *Bacillus anthracis*?
- b. How sensitive is the assay when target cells or spores are applied directly to the consumable sampler?
- c. What is the effect of some common nontarget (interferent) materials on the performance of the assay when target cells or spores are used?

3. SCOPE

This study is the first part of an overall plan to test the sensitivity, specificity, and resistance to interferents of three assays designed for use in the Bio-Seeq[®] instrument. The probe and primer sets in the assays are designed to each detect one of the following threat agents: *Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*.

4. METHODS AND MATERIALS

4.1 Instrument Description.

Smiths Detection - Edgewood, Inc. is the manufacturer of the Bio-Seeq[®] instrument (Fig. 1). Two Bio-Seeq[®] units (s/n 115, and another unit dubbed "thermal paste") were provided to ECBC by SDE. Each unit contains six thermocycler modules that are independently programmable and operable. Each module can be run separately. The instrument has an LCD panel that allows an operator to enter, store, and run programs that instruct each module to heat and cool a PCR reaction tube to a specified temperature for a specified length of time, within the operating parameters of the instrument. A user can also monitor and gather data from each module independently using a PC that is running proprietary software developed by SDE (latest version at this writing is 1.21). When connected to a PC, the instrument will graphically display the development of the fluorescent signal generated by a PCR assay reaction in real time, as well as the signal generated by internal positive control reagents, which are contained in the reagent beads.

The operator conducts an assay using a consumable sampling and reaction tube assembly (a "consumable", Fig. 2). The consumable is supplied in two assembled pieces. One piece consists of a buffer cup housing with a small handle that contains the buffer cup. Buffer is contained in the cup by a thin breakable plastic film. The second piece consists of a housing called a reagent base, which contains a hollow plunger tipped with a porous swab. Within the hollow plunger are three dried beads, which contain the assay reagents, and an inert mixing bead. A hole at the end of this piece leads into the attached clear plastic reaction tube.

To conduct an assay, the operator would remove the two pieces from their packaging, and apply the swab-tipped end of the reagent base to the surface to be sampled. The swab tip would then be inserted into the open end of the buffer cup holder, and twisted. Threads on the buffer cup housing and the reagent base cause the swab tip to press against and break the film containing the buffer, which carries sample material through the porous tip and into the interior of the hollow plunger. By allowing the buffer to reside in the plunger for 90 seconds period the buffer dissolves the reagent beads. The operator then shakes the device for 20 seconds causing the mixing bead to mix the sample, the buffer, and the assay reagents. The operator "whips" the assembled consumable to draw the aqueous reaction mixture into the reaction tube,

which is then inserted into one of the six PCR modules in the Bio-Seeq[®] instrument. The operator then starts the program of PCR temperatures and durations appropriate to the particular assay reagents for that test.

4.2 Bacterial Strains, Culture Methods, and DNA Isolation.

The strains used in this study are listed in Table 1. All strains listed were obtained from an in-house culture collection at ECBC; most are available from ATCC (Manassas, VA) or the Bacillus Genetic Stock Center (The Ohio State University, Columbus, OH). All strains used in this study were nonpathogenic, and were handled according to BSL-2 practices. Strains of *Bacillus* spp. were grown for DNA isolation in liquid Nutrient Broth (Difco), prepared according to the manufacturer's instructions. Bacteria were grown overnight at 30°C in a shaking incubator at 220 rpm. Cells were harvested by centrifugation. DNA was extracted from bacterial cells with QIAGEN DNeasy mini spin columns and reagents (QIAGEN, Valencia, CA), using the manufacturer's instructions for the isolation of total genomic DNA from gram-positive bacteria. DNA was quantified and tested for purity by measuring the absorbance spectrophotometrically at 260 nm and 280 nm. DNA from all isolations was dissolved and diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

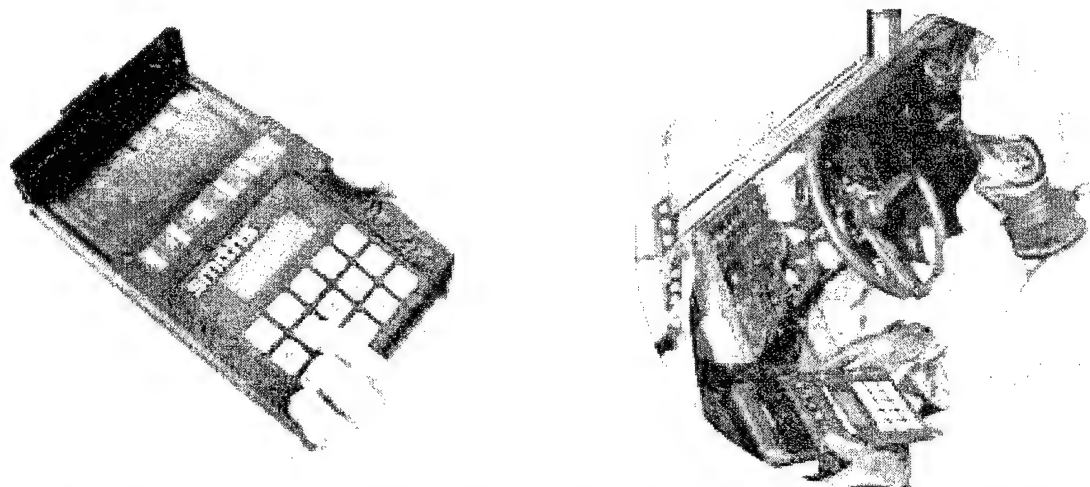


Figure 1. Bio-Seeq[®] Instrument. Left, the lightshield is open, revealing the openings for 6 Bio-Seeq[®] tubes. Right, an illustration of the concept of operation. An operator, wearing protective equipment in a potentially hazardous environment, is about to conduct an assay.

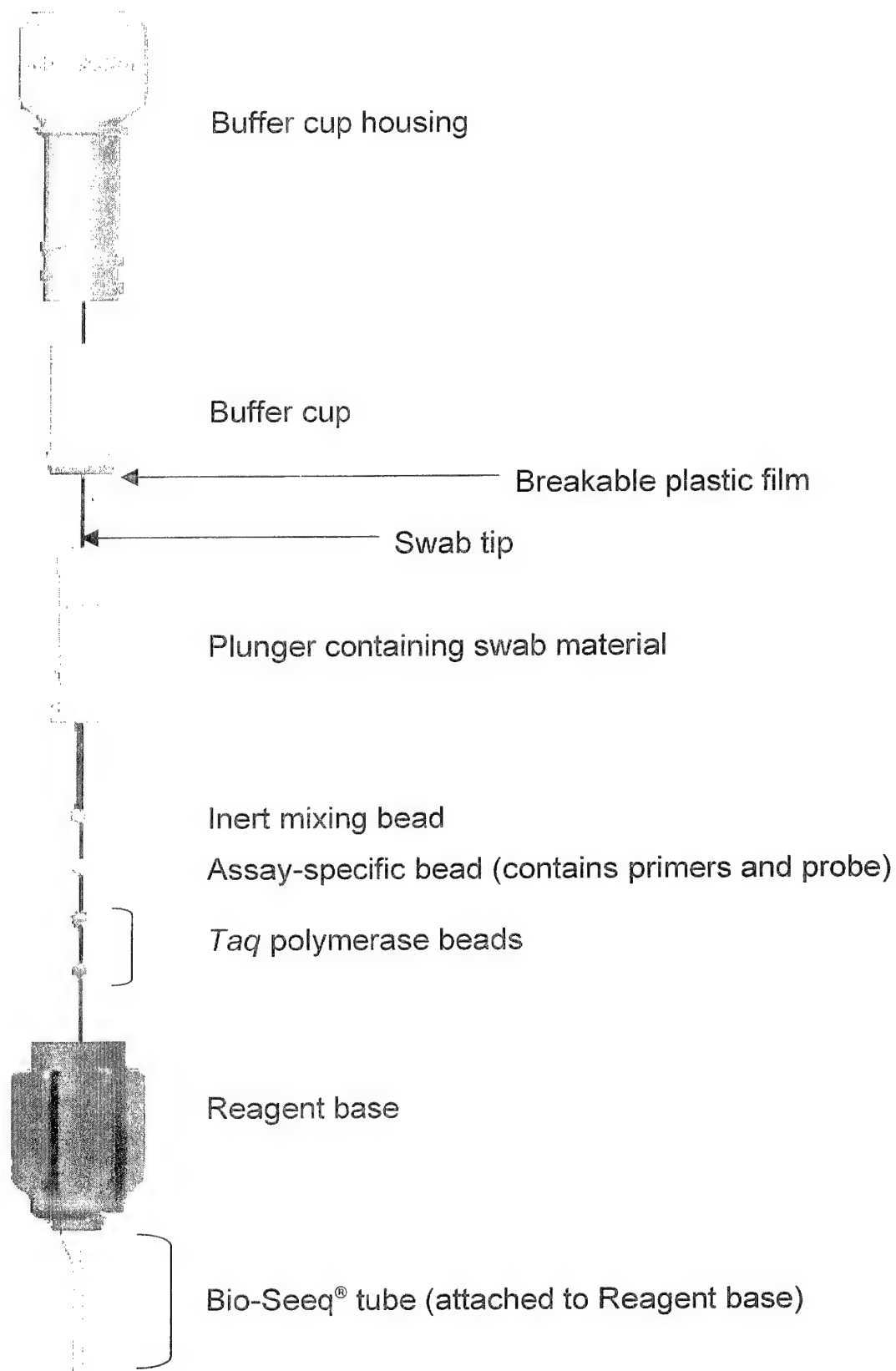


Figure 2. Consumable Sampling Device with Reaction Tube for the Bio-Seeq®.

Table 1. Bacterial Species and Strains Used in This Study.

<i>Bacillus anthracis</i>	<i>Bacillus thuringiensis</i>	<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>
ΔAmes	4A2	ATCC 11778	ATCC 23059 ^a
ΔSterne	4E3	ATCC 13824	3A8
ΔNH-1	4F4	ATCC 6464	YB886 ^b
VNR1-Δ1	405	ATCC 12826	var. niger ^c
NNR1-Δ1	401	6E1	1031
NNR-1	4C2	1122	
ANR-1	4J4	1219	
	4T4	631	
	ATCC 10792		
	var. kurstaki		

Other *Bacillus* species/strains:

B. mycoides ATCC 6462
B. megaterium ATCC 14581
B. sphaericus

^a also called W23

^b also BGSC # 1A304, a derivative of strain 168

^c also called *B. globigii* or "BG"

The number of PCR assay targets was estimated to be one per genome copy. The number of copies of a *Bacillus* genome per unit mass was calculated in the manner described below. Few genomes of *Bacillus* spp. have been fully sequenced; however, we have taken an average of the genome sizes of two *Bacillus anthracis* strains that have been sequenced (Ames, and A2012) as a reasonable estimate (see <http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html>). Because *B. cereus* and *B. thuringiensis* are very similar to *B. anthracis*, we applied the estimated genome size for *B. anthracis* (5.17 Mbp) to the other bacilli as well.

To calculate the mass of 1000 genomes:

1. Calculate Molecular Weight (MW):
 $= 5.17 \text{ Mbp} \times 660 \text{ g/mole/bp} = 3.4122 \times 10^9 \text{ g/mole of genomes}$
2. Calculate mass of 1000 genomes:
 $3.4122 \times 10^9 \text{ g} = 6.023 \times 10^{23} \text{ genomes}$
 $5.6653 \times 10^8 \text{ g} = 10^{23} \text{ genomes} \quad (\text{divide by } 6.023)$
 $5.6653 \times 10^{-12} \text{ g} = 10^3 \text{ genomes} \quad (\text{divide by } 10^{20})$

Therefore, 1000 copies of a *Bacillus* genome weighs approximately 5.6 picograms.

4.3

Spore Preparation.

Cells of *B. cereus* strain 1219, *B. thuringiensis* var. *kurstaki*, and *B. anthracis* strain NNR-1 were spread on several plates of solid sporulation medium (per liter, 3 g yeast extract, 2 g tryptone, 2 g agar, 1 ml 1% aqueous $MnCl_2$, and 23 g Lemko agar) and incubated 4-6 days at 30°C. The extent of sporulation was determined by examining samples from each set of plates under a phase contrast microscope (1000x, with an oil immersion lens). Digital images were captured and stored (Fig. 3). All strains were more than 99% sporulated after 5 days of incubation. Strain ANR-1 was also plated on sporulation medium; however, this strain did not form phase-bright spores and after 10-12 days on the solid medium was no longer viable. Spores were removed from plates by gentle scraping and suspended in 10 mM Tris HCl, pH 7.4 (Sigma Co., St. Louis, MO). Spores were diluted in the same Tris buffer for enumeration by plate counting and for use in the Bio-Seeq[®] assay.

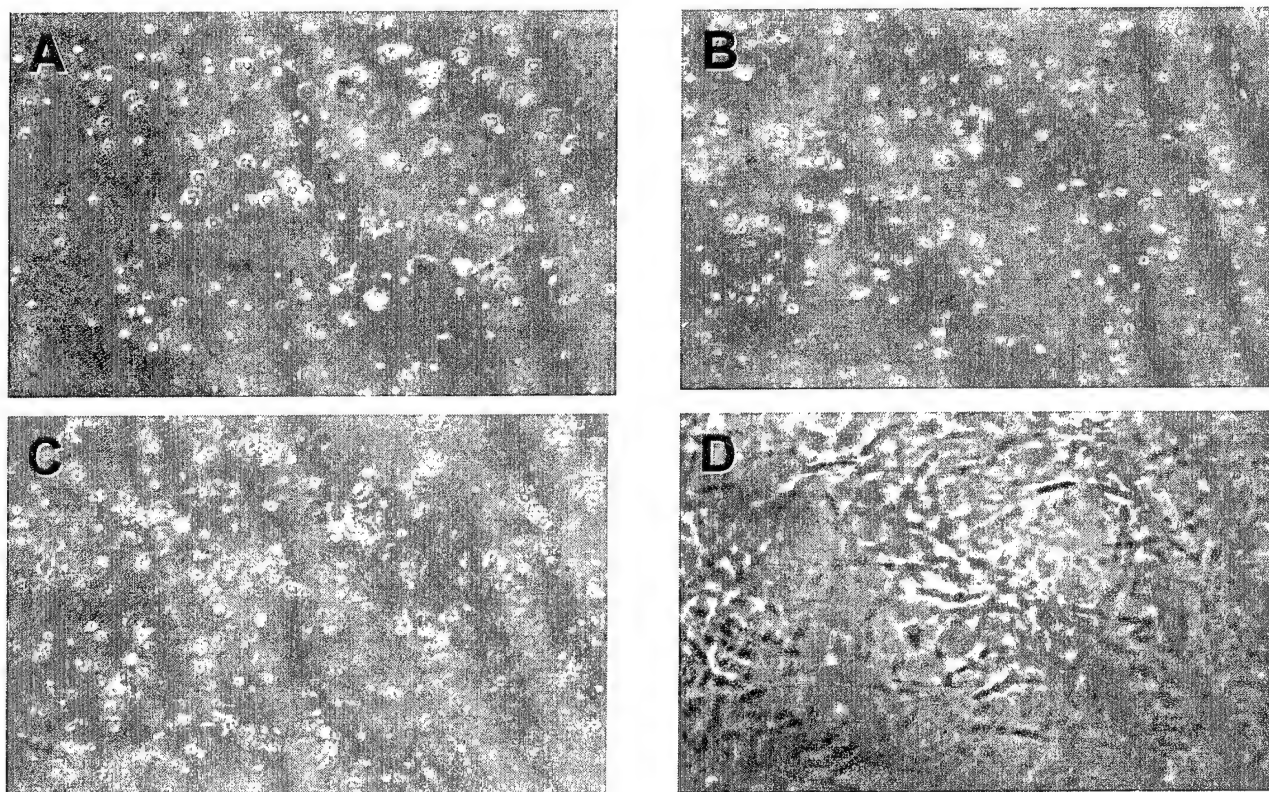


Figure 3. Spores of *Bacillus* Species/Strains Used in the Sensitivity and Specificity Testing of the Consumable Sampling Device. A, *B. anthracis* NNR-1; B, *B. cereus* 1219; C, *B. thuringiensis* var. *kurstaki*; D, *B. anthracis* ANR-1. Note the lack of phase-bright spores in the ANR-1 culture.

4.4 Choice of Interferents.

Interferents were chosen by the marketing staff at SDE, in consultation with their marketing department. Four common materials were selected: coffee creamer (Domino Non-Dairy Creamer), baking powder (Rumford brand, Clabber Girl Inc., Terre Haute IN), wheat flour (America's Choice unbleached), and corn starch (Giant brand). Each material is found in most home kitchens and in many workplaces (especially coffee creamer). They may either be found on surfaces that have been contaminated with *Bacillus anthracis* spores, or used by the perpetrator of a hoax, as it is widely believed that weaponized spores have the appearance of a white powder. All interferents were obtained by SDE staff at local supermarkets. Aliquots of each interferent were weighed and placed into 15 ml disposable plastic tubes before being supplied to the ECBC technical staff. Interferents were dissolved or suspended in nuclease-free, molecular biology grade water (Gibco BRL / Invitrogen, Carlsbad, CA) before use.

4.5 Assay Set-Up and Data Collection.

Assays were prepared in the following three ways:

a. To test the specificity and inclusivity of the probe and primer set using isolated total genomic DNA (data in Tables 2 and 3): for two reactions, a single dried bead containing the *B. anthracis* probe, primers, and internal control reagents was placed in a 1.5 ml microcentrifuge tube with two Ready-to-Go PCR reagent beads (containing *Taq* polymerase, dNTPs, and buffer) (Amersham Biosciences, Piscataway, NJ) and 48 μ l nuclease-free water. When the reagent beads were dissolved, the mixture was divided into two 24 μ l aliquots. One microliter of DNA suspension was added to each reaction mixture (total volume 25 μ l). Each reaction mixture was transferred to a Bio-Seeq[®] reaction tube, capped and covered with Parafilm, and gently centrifuged to draw the reaction mixture into the tube. Each tube was then placed into a separate PCR module in the Bio-Seeq[®] instrument and the thermocycling was started according to the manufacturer's instructions.

Table 2. Detection of DNA from pXO¹⁺ Strains of *B. anthracis*.

Target DNA ^a	No. positive/total ^b	Ct values ^c
ANR-1	3/3	32, 32, 32
NNR-1	3/3	35, 34, 34
plasmid pXO1	5/7	39, 42, 42, 45, 47
synthetic target	2/2	33, 34
NTC	0/3	N/A

^a1000 copies per reaction. NTC, no-template control (negative control).

^breactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

Table 3. Detection of DNA from pXO¹⁻ Strains of *B. anthracis* and Other Species.

Target DNA ^a	No. positive/total ^b	Ct values ^c
<i>B. anthracis</i> :		
ΔAmes	0/3	N/A
ΔSterne	1/3	47
ΔNH-1	0/3	N/A
VNR1-Δ1	0/3	N/A
NNR1-Δ1	0/3	N/A
<i>B. thuringiensis</i>		
4A2	0/3	N/A
4E3	0/3	N/A
4F4	0/3	N/A
405	0/3	N/A
401	0/3	N/A
4C2	0/3	N/A
4J4	0/3	N/A
4T4	0/3	N/A
ATCC 10792	0/3	N/A
var. kurstaki	0/3	N/A
<i>B. cereus</i>		
ATCC 11778	0/3	N/A
ATCC 13824	0/3	N/A
ATCC 6464	0/3	N/A
ATCC 12826	0/3	N/A
6E1	0/3	N/A
1122	0/3	N/A
1219	0/3	N/A
631	0/3	N/A
<i>B. subtilis</i>		
ATCC 23059	0/3	N/A
3A8	0/3	N/A
YB886	0/3	N/A
var. niger (<i>B. globigii</i>)	0/3	N/A
1031	0/3	N/A
<i>B. mycoides</i> ATCC 6462	0/3	N/A
<i>B. megaterium</i> ATCC 14581	0/3	N/A
<i>B. sphaericus</i>	0/3	N/A
synthetic target	20/20	7 @ 33, 11 @ 34, 2 @ 35
NTC	0/3	N/A

^a 100,000 copies per reaction, except for synthetic target (1000 copies). NTC, no-template control (negative control).

^b reactions run on either of two instruments.

^c N/A, not applicable. Values are reported only for positive assay results.

b. To test the limit of detection of the assay using viable spores as target, in an assembled consumable sampling device (a "consumable"; Fig. 2) (data in Tables 4, 5, and 6): a single *B. anthracis* reagent bead and two Ready-to-Go PCR reagent beads were placed in a swab-tipped plunger with an inert mixing bead, and the plunger was inserted snugly into the reagent base end of a consumable with a reaction tube affixed. Five μ l of a spore suspension (or DNA suspension or Tris buffer, in the control experiments) was spotted onto the surface of the swab tip. The consumable portion with the sample-bearing plunger, the reagents, and the reaction tube tip were then assembled with the portion containing the buffer cup. Twisting the two halves together broke the film on the buffer cup and allowed the sample and the reagent beads to be suspended in the buffer.

Assembled consumables were held inverted at room temperature for 90 sec to allow the beads to dissolve, then shaken for 20 sec to complete the dissolving and mix the reagents with the sample. The consumable was then "whipped" with a snapping motion of the wrist to drive a portion of the reaction mixture into the reaction tube. The reagent tube tip of the consumable was then inserted into one of the six PCR modules in the Bio-Seq[®] instrument.

c. To test the effect of interferent materials on the ability of the assay to detect *B. anthracis* spores (data in Tables 7-10): reagent beads were inserted into a swab-tipped plunger, the plunger was assembled with the reagent base end of a consumable, and the swab was spotted with target suspension as described above. However, interferent powders in suspension were not easily or accurately delivered by pipette to the swab tip, making necessary the following variation. One hundred thirty microliters of an aqueous suspension of an interferent was added to empty unsealed buffer cups, which was then gently assembled with the swab-tipped plunger (to which a sample target had already been added). The buffer cup holder was then placed around the buffer cup and swab-tipped plunger. The entire consumable was then assembled, interferent-containing buffer dissolved the reagents in the plunger, and the rest of the assay was performed as described above.

Table 4. Detection of Nontarget *Bacillus* Spores Applied to the Consumable Sampling Device.

No. spores added	No. positive/total ^b	Ct values ^c
<i>B. thuringiensis</i> var. <i>kurstaki</i> 10 ⁶	0/3	N/A
<i>B. cereus</i> 1219 10 ⁶	0/3	N/A
synthetic target (5000 copies) ^a	2/2	35, 40

^a5000 copies of target applied to the consumable swab tip.

^breactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

Table 5. Detection of NNR-1 Spores Using the BA Reagent Bead Set^a.

No. spores added	No. positive/total ^b	Ct values ^c
<i>B. anthracis</i> NNR-1		
4.5 x 10 ⁵	3/3	24, 25, 25
10 ⁵	3/3	26, 27, 28
10 ⁴	3/3	29, 30, 31
10 ³	3/3	33, 33, 34
10 ²	3/3	36, 44, 48
10 ¹	0/3	N/A
10 ⁰	0/3	N/A
0	0/3	N/A

^aReaction mixtures prepared in a microcentrifuge tube and placed directly into a Bio-Seeq[®] reaction tube, without passing through the complete, assembled consumable sampling device.

^breactions run on either of two instruments. Date of experiment: 18 November 2003.

^cN/A, not applicable. Values are reported only for positive assay results.

Table 6. Detection of NNR-1 Spores Applied to the Consumable Sampling Device.

Date	No. spores added	No. positive/total ^b	Ct values ^c
	<i>B. anthracis</i> NNR-1		
11/13/03	10 ⁶	3/4	27, 30, 30
11/13/03	10 ⁵	3/3	31, 33, 37
11/13/03	10 ⁴	2/3	35, 36
11/18/03	10 ⁴	3/3	32, 36, 36
11/18/03	5x10 ³	3/3	32, 26, 38
11/18/03	10 ³	1/3	43
11/13/03	10 ³	0/3	N/A
11/13/03	10 ²	1/3	35
11/13/03	10 ¹	1/3	37
11/13/03	10 ⁰	0/3	N/A
11/13/03	0	0/3	N/A
11/18/03	0	0/3	N/A
11/13/03	synthetic target (1000 copies)	1/4	48
11/13/03	synthetic target (2000 copies)	1/3	37
11/13/03	synthetic target (tube only) ^a	3/3	34, 35, 35

^a1000 copies of synthetic target per reaction, prepared in a microcentrifuge tube and placed directly into a Bio-Seeq[®] reaction tube, without passing through the complete, assembled consumable sampling device.

^breactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

5. RESULTS AND DISCUSSION

5.1 Specificity of Reagents for *Bacillus* Species.

The assay for *B. anthracis* detects the presence of sequences resident on the plasmid pXO1; strains containing this plasmid therefore should be detected by the assay. The *B. anthracis* strains used in this study that contain pXO1 are NNR-1 and ANR-1. All other strains listed have been cured of pXO1 and/or pXO2. Total genomic DNA was prepared from each species and strain listed in Table 1, as described above.

To determine whether the assay accurately identified *B. anthracis* strains containing pXO1, we diluted preparations of genomic DNA from strains NNR-1 and ANR-1 to a concentration of 1000 genome copies per microliter. One microliter of each DNA suspension was added to 24 μ l of reaction mixture as described above, placed in a reaction tube and inserted into the instrument. As positive controls, we also assembled reactions containing 1000 copies of the synthetic target DNA designed to match the assay reagents, and assays containing 1000 copies of purified isolated pXO1 DNA. The PCR thermocycler profile was run as pre-programmed by SDE.

When prepared as described above, the assay detected 1000 copies of genomic DNA containing pXO1 sequences (from both strains ANR-1 and NNR-1), as well as 1000 copies of the synthetic target (Table 2). Assays detecting 1000 copies of pXO1 plasmid DNA gave variable results (5 out of 7 assays reported a detection event).

The specificity of the assay was also tested by including large amounts of total genomic DNA from *Bacillus anthracis* strains that do not contain pXO1, and from other species of *Bacillus* (Table 3). None of the other strains tested reacted positively (a single positive reaction with *B. anthracis* strain Δ Sterne was attributed to the presence of an air bubble in the reaction tube during the assay).

5.2 Limits of Detection Using the Consumable Sampling Device.

After observing that NNR-1 and ANR-1 genomic DNA gave positive results with the assay, we grew cells of both strains on solid sporulation medium to generate spores for testing with the consumable. In addition, we grew cells of *B. thuringiensis* var. *kurstaki* and *B. cereus* strain 1219 for use as negative controls. However, spores of strain ANR-1 failed to form after more than 10 days of incubation, and the cell material that remained on the agar medium was non-viable at that time (Fig. 1). Therefore, spores of NNR-1 alone were used as the target strain for limit of detection (LOD) experiments. The assay including the consumable was also tested against two other species of *Bacillus*, neither of which was detected by the assay, as anticipated (Table 4).

Laboratory assays for the detection of NNR-1 spores, using the reagent bead set (without the consumable), were prepared as described in Materials and Methods. A suspension of spores was serially diluted in sterile 10 mM Tris HCl, pH 7.4 (Sigma Co., St. Louis, MO), and aliquots containing spores (or buffer alone) were added to PCR reaction mixtures prepared in microcentrifuge tubes, then placed in Bio-Seeq® reaction tubes. The reagent bead set, prepared as described, allowed the detection of as few as 10^2 spores per assay (Table 5).

To examine the sensitivity of the assay for *B. anthracis* NNR-1 when the reagent bead set is incorporated into the consumable sampling device, we performed the assays at several spore concentrations, applying spores to the consumable by spotting five-1 μ l aliquots around edge of the swab tip, where the swab tip is most porous. The data (Table 6) indicate that the assay for NNR-1 spores has a LOD of approximately 10^3 to 5×10^3 spores per assay using the consumables.

In aggregate, the data from Tables 4 and 6 also indicate that the assay is less sensitive for the detection of synthetic target when the target is applied to the consumable. Five thousand copies of target gave positive results consistently (Table 4), whereas 1000 and 2000 copies did not (Table 6). One thousand copies of synthetic target were sufficient to give a positive result when the consumable was not used (Tables 2 and 6).

5.3 Effects of Interferents in Laboratory Tests of the BA Assay.

Several concentrations of each inhibitor were used to test the effect of each on the ability of the assay to detect spores of NNR-1. SDE staff determined the concentrations to be tested based upon work done at the SDE labs. The goal was to test a set of concentrations of each inhibitor that would bracket the minimum inhibitory concentration. All inhibitors were dissolved/suspended in nuclease-free water. All spore suspensions were diluted in 10 mM Tris HCl, pH 7.4. Tris diluent was used in place of spores in all of the negative controls.

Cornstarch had little effect on the performance of the assay (Table 7). In this study, we did not reach a concentration of cornstarch that was completely inhibitory to the assay. The inhibitory amount per assay, in the presence of 10^4 spores, is in excess of 7.5 mg per assay.

Similarly, coffee creamer, of all four of the interferents, had the least effect on the performance of the assay (Table 8). No concentration of coffee creamer was tested which completely inhibited the assay at the lowest spore concentration detected (10^4 spores per assay). The minimum inhibitory concentration of coffee creamer in this study was in excess of 15 mg per assay.

Table 7. Detection of NNR-1 Spores Applied to the Consumable Sampling Device in the Presence of Cornstarch.

Date	Amount per assay ^a	No. spores added	No. positive/total ^b	Ct values ^c
11/13/03	0.75 mg	10 ⁶	3/3	22, 26, 34
		10 ⁵	2/3	31, 34
		10 ⁴	3/3	35, 36, 42
		synthetic target (3000 copies)	1/1	37
		cornstarch and water only	0/1	N/A
		NTC	0/1	N/A
11/13/03	2.5 mg	10 ⁶	3/3	26, 27, 29
		10 ⁵	3/3	29, 32, 36
		10 ⁴	2/3	28, 37
		cornstarch and water only	0/2	N/A
		NTC	0/1	N/A
11/17/03	5 mg	10 ⁶	3/3	27, 29, 33
11/17/03		10 ⁵	3/3	33, 33, 36
11/18/03		10 ⁴	3/3	37, 37, 37
11/17/03	7.5 mg	10 ⁶	3/3	28, 29, 35
11/17/03		10 ⁵	3/3	34, 34, 47
11/18/03		10 ⁴	2/3	35, 41

^aTotal amount of interferent introduced into the consumable buffer cup in 130 µl of water. NTC, no-template control (negative control).

^bReactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

An examination of the consumable suggests that the amounts of coffee creamer and cornstarch needed to partially inhibit the performance of the assay are far in excess of the amounts that would adhere to the swab tip when sampling a surface. We conclude from these results that neither coffee creamer nor cornstarch significantly inhibits the assay in amounts likely to be introduced into the interior of the consumable.

However, both flour and baking powder inhibited the assay in quantities far less than coffee creamer or cornstarch. Amounts of flour far less than 1 mg significantly inhibited the assay (Table 9). This inhibition is probably due, at least in part, to the presence of high molecular weight polysaccharides (mostly starches, in white flour) and protein (gluten). Both of these components contribute to the tendency of flour to form a sticky suspension at higher concentrations (eventually approaching the consistency of dough). The minimum inhibitory concentration of flour in this study is between 0.1 and 0.01 mg per assay at the lowest concentration of spores added (10⁴). Only two out of three assays showed a positive response in the presence of 0.1 mg flour per assay.

Table 8. Detection of NNR-1 Spores Applied to the Consumable Sampling Device in the Presence of Coffee Creamer.

Date	Amount per assay ^a	No. spores added	No. positive/total ^b	Ct values ^c
11/11/03	2.5 mg	10 ⁶	3/3	26, 26, 28
		10 ⁵	3/3	27, 29, 29
		10 ⁴	3/3	32, 32, 33
		coffee creamer and water only	0/2	N/A
		NTC	0/1	N/A
11/11/03	5 mg	10 ⁶	3/3	25, 27, 28
		10 ⁵	3/3	25, 28, 28
		10 ⁴	3/3	31, 35, 41
		coffee creamer and water only	1/2	48
		NTC	0/1	N/A
11/17/03	10 mg	10 ⁶	2/3	27, 29
11/17/03		10 ⁵	3/3	20, 31, 36
11/18/03		10 ⁴	3/3	32, 32, 33
11/17/03	15 mg	10 ⁶	2/3	30, 30
11/17/03		10 ⁵	2/3	33, 46
11/18/03		10 ⁴	2/3	35, 35

^aTotal amount of interferent introduced into the consumable buffer cup in 130 μ l of water. NTC, no-template control (negative control).

^bReactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

Even smaller amounts of baking powder were inhibitory to the assay (Table 10). The minimum inhibitory concentration of baking powder observed in this study was between 0.025 and 0.0025 mg per assay when 10⁴ spores were detected. At 0.025 mg baking powder per assay, only two out of three assays gave a positive result. We hypothesize that the inhibition by baking powder is the result of some combination of changes in the pH of the reaction solution brought about by the acid-base reaction of baking powder components with water and generation of CO₂ by the same reaction. Gas bubbles in Bio-Seeq[®] tubes are perhaps the single greatest cause of experimental artifacts (false positive and false negative results). Another component of some baking powders (including that used in this work), calcium acid phosphate (Ca(H₂PO₄)₂) contains pyrophosphate, which may also inhibit *Taq* polymerase because it is a product of the DNA polymerization reaction. Some flours ("self-rising" flour) contain baking powder; the effect of these two interferents combined in this fashion was not examined in this study.

Table 9. Detection of NNR-1 Spores Applied to the Consumable Sampling Device in the Presence of Wheat Flour.

Date	Amount per assay ^a	No. spores added	No. positive/total ^b	Ct values ^c
11/14/03	0.01 mg	10 ⁴ Flour and water only NTC	3/3 0/2 0/1	39, 39, 43 N/A N/A
11/14/03	0.1 mg	10 ⁶ 10 ⁵ 10 ⁴ Flour and water only NTC	3/3 3/3 2/3 0/2 0/1	25, 25, 31 31, 33, 35 32, 38 N/A N/A
11/17/03	0.2 mg	10 ⁶	3/3	20, 34, 34
11/17/03		10 ⁵	3/3	36, 38, 49
11/18/03		10 ⁴	0/3	N/A
11/17/03	0.3 mg	10 ⁶	2/3	31, 40
11/17/03		10 ⁵	3/3	39, 40, 46
11/18/03		10 ⁴	1/3	25

^aTotal amount of interferent introduced into the consumable buffer cup in 130 µl of water. NTC, no-template control (negative control).

^bReactions run on either of two instruments.

^cN/A, not applicable. Values are reported only for positive assay results.

Table 10. Detection of NNR-1 Spores Applied to the Consumable Sampling Device in the Presence of Baking Powder.

Date	Amount per assay ^a	No. spores added	No. positive/total ^b	Ct values ^c
11/18/03	0 mg	10 ⁴	3/3	21, 32, 36
11/14/03	0.0025 mg	10 ⁴ Baking powder and water only NTC	3/3 0/2 0/1	34, 34, 36 N/A N/A
11/14/03	0.025 mg	10 ⁶ 10 ⁵ 10 ⁴ Baking powder and water only NTC	3/3 3/3 2/3 0/2 0/1	23, 24, 29 30, 30, 32 28, 44 N/A N/A
11/17/03	0.05 mg	10 ⁶	2/3	28, 30
11/17/03		10 ⁵	0/3	N/A
11/18/03		10 ⁴	1/3	42
11/17/03	0.075 mg	10 ⁶	1/3	20
11/17/03		10 ⁵	1/3	50

^aTotal amount of interferent introduced into the consumable buffer cup in 130 µl of water. NTC, no-template control (negative control).

^bReactions run on either of two instruments. N/D, not done.

^cN/A, not applicable. Values are reported only for positive assay results.

6.

CONCLUSIONS

The Bio-Seeq[®] instrument and reagents for *B. anthracis* assays correctly identified *B. anthracis* strains containing the target DNA sequences, and did not react with over 30 other species and strains of *Bacillus*. Under laboratory conditions, the sensitivity of the assay was observed to be 100 spores per assay (without consumable; the observed LOD while using the consumable was 1000-5000 spores). The addition of coffee creamer or cornstarch had little effect on the performance of the assay under the conditions tested; however, both flour and baking powder adversely affected assay performance, reducing the sensitivity several orders of magnitude when the assay was performed using the consumable sampling device.

It must be pointed out that no sample preparation or DNA clean-up steps were involved in the assays performed in this study. It is likely that the target sequences being detected were present in DNA clinging to the outside of spores, or carried along in the spore preparation from the initial spore isolation, since no steps were taken to break open spores to release the DNA contained inside. Effective sample processing (resulting in recovery of most of the sample DNA in pure form) should increase the sensitivity of the assay and remove most of the problems caused by the presence of interferents. Future testing should include:

- use of the sampling tip to pick up spores from a variety of surfaces
- tests against a larger panel of DNA isolated from pXO1⁺ *B. anthracis* or live virulent spores.

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